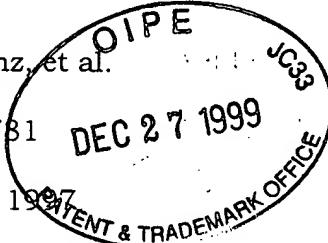


Docket No. 46745 (1758)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: J. Weidanz, et al.  
SERIAL NO.: 08/813,781  
FILED: March 7, 1997  
FOR: FUSION PROTEINS COMPRISING BACTERIOPHAGE COAT PROTEIN AND A SINGLE-CHAIN T CELL RECEPTOR

EXAMINER: M. Lubet  
GROUP: 1644



THE HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS  
WASHINGTON, DC 20231

SIR:

**DECLARATION PURSUANT TO 37 CFR 1.131**

The undersigned declare as follows:

1. We are co-inventors of the above-identified application (hereafter the "subject application"). Jon A. Weidanz and Hing Wong are Senior Scientists with Sunol Molecular Corporation, Miramar Florida. Hing Wong is currently President and CEO of that corporation.

2. As we understand it, the subject application discloses and claims, among other things, fusion proteins in which bacteriophage coat protein is covalently linked (ie. fused) to a soluble single-chain T-cell receptor. As one example, the subject application discloses and claims, a single-chain T-cell receptor linked to a bacteriophage VIII coat protein ("single chain fusion protein").

3. We have reviewed the Patent Office Action ("Office Action") dated June 23, 1999 issued in connection with the subject application. As we understand the Office Action, the patent Examiner rejected claims 1, 2, 4, 7-8, 14, 18, 19, 20, 61 and 67 as obvious in view of WO 96/18105 ("Strominger") taken with other references

cited in the Office Action. We understand that Strominger is a PCT application having an issue date of 13 June 1996.

4. We have read Strominger. As we understand it, Strominger discloses a specific single-chain T-cell receptor that binds to an MHC peptide ligand.

5. The invention described and claimed in the subject application was conceived and reduced to practice in the United States prior to June 1996.

6. For example, well before June 1996, we recognized that it was possible to make a recombinant T-cell receptor (TCR) protein in which a V-alpha chain was fused to a V-beta C-beta chain by a peptide linker sequence. It was also recognized before the June 1996 date that a bacteriophage coat protein could be fused to the V-beta C-beta chain to produce the single-chain fusion protein. As understood, use of the bacteriophage gene VIII coat protein could substantially improve features of the fusion protein. As disclosed throughout the subject application, that recognition helped us make and use the single-chain fusion protein.

7. The claimed single-chain fusion protein was made in the United States well before Strominger's June 1996 publication date.

8. For example, conception of the single-chain fusion protein was accomplished well before Strominger's publication date. Attached as **Exhibit 1** is a true and accurate copy, with dates deleted, of notes made by one of us before June 1996. For example, see pages 1 and 2 of the notes in which is shown, among other things, drawings of single-chain fusion protein. Also indicated in the notes, among other things, are steps for making vector encoding peptide linker sequence fused to the bacteriophage gene VIII protein. The vector is referred to as pKC27. Construction and use of the pKC27 vector is further exemplified by the subject application. For

instance, see Example 2 and particularly part E which discloses work performed well before the June 1996 date. See also Figure 2 of the application (outlining production of pKC27 and other vectors).

9. One of us prepared sequence encoding TCR V-alpha chain well before Strominger's June 1996 publication date. Attached as **Exhibit 2** is a true and accurate copy of notes, with dates deleted, prepared by one of us well before that date. The notes show, among other things, successful production of sequence encoding the V-alpha chain. These manipulations were performed well before the June 1996 date and are described throughout the subject application, for instance, see Example 1.

10. Well before the June 1996 publication date of Strominger, one of us fused sequence encoding the V-alpha chain into the pKC27 vector. That manipulation led to production of pKC42 vector. The pKC42 vector particularly encodes sequence in which the V-alpha chain is fused to the peptide linker. That linker is fused to the bacteriophage gene VIII protein. Attached as **Exhibit 3** is a true and accurate copy of notes made by one of us, with dates deleted, describing steps taken to make the pKC42 vector among other things. These steps were undertaken well before the June 1996 date and are further exemplified in the subject application.

For instance, see Example 2 and particularly section E. See also Figure 2 of the subject application (outlining production of pKC42 from the pKC27 vector).

11. We made vectors that encode the TCR V-beta C-beta chain well before Strominger's June 1996 publication date. Attached as **Exhibit 4** is a true and accurate copy of notes made by one of us, with dates deleted, that shows, among other things, steps taken to make pKC30 vector. These manipulations were conducted well before the June 1996 date and are further exemplified in the subject application. See Figure 2, for example.

12. In experiments performed well before Strominger's June 1996 publication date, we made vectors that encode the V-beta C-beta chain fused to the bacteriophage gene VIII protein. Attached as **Exhibit 5** is a true and accurate copy of a note made by one of us, with dates deleted, which shows, among other things, manipulation of a vector called pKC34.3. The pKC34.3 vector is a specific isolate of pKC34 and it is further exemplified in Figure 2 of the subject application (see the step for making pKC44 from the pKC42). The note particularly describes treatment of pKC34.3 with restriction endonucleases to isolate a fragment encoding the V-beta C-beta chain fusion protein. Steps taken to make the pKC34.3 vector were performed well before the June 1996 date.

13. Well before Strominger's June 1996 publication date, we inserted sequence encoding the V-beta C-beta bacteriophage gene VIII fusion protein into the pKC42 vector. Attached as **Exhibit 6** is a true and accurate copy of notes made by one of us, with dates deleted, showing, among other things, production of the pKC44 vector (encodes single-chain fusion protein). In particular, pages 1-5 of the notes show manipulation of specific pKC42 vectors (42.1, 42.2, and 42.3) and use of those vectors as recipients of sequence encoding the V-beta C-beta bacteriophage gene VIII construct. These results are further exemplified by the subject application. For instance, see Example 2 and particularly section E. See also Figure 2 of the application (showing production of pKC44 from pKC42).

14. We made the single-chain fusion protein in the United States well before the June 1996 publication date of Strominger.

15. For example, attached as **Exhibit 7** is a true and accurate copy of notes made by one of us, with dates deleted, that shows, among other things, manufacture of the single-chain fusion protein well before Strominger's June 1996 date. In particular, pages 1-4 of the notes show expression of the single-chain fusion protein

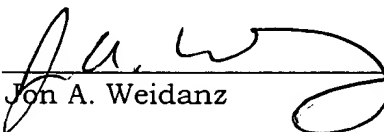
encoded by pKC44 as evidenced by a Western Blot. Pages 5 and 6 of the notes show purification of that protein using an immuno-affinity column. These manipulations are further exemplified by the subject application. For instance, see Examples 4, 5, 6 and Figures 7-13 which disclose work performed well before June 1996.

16. We hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

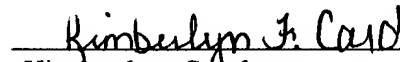
Date: 12/14/99

  
Hing C. Wong

Date: 12/1/99

  
Jon A. Weidanz

Date: 12/14/99

  
Kimberlyn Card

Objective: To begin work on a single chain construct  $L = \text{linker}$   $S = \text{gene S}$   
 as an alternative to the current Fab constructs:

pkc3.1 pkc3.6

The fusion protein may not be made efficiently as the 2nd protein after a single promoter. A single chain construct may overcome this type of problem. The two proteins will be seen as a single protein connected together by a linker sequence.

Step 1: Cut vector with gene VIII to allow linker as insert.

Method/Materials: pkc15.1 DNA prep @ 390 ng/ $\mu$ l  
 Spe I @ 15 U/ $\mu$ l  
 Xho I @ 20 U/ $\mu$ l  
 BSA (1:10)  
 BSA<sup>100</sup> Buffer  
 purified water

Precipitate cut 10  $\mu$ g of pkc15.1 set up digest:

25.3  $\mu$ l pkc15.1 (10  $\mu$ g)  
 3.3  $\mu$ l Spe I  
 2.5  $\mu$ l Xho I  
 12  $\mu$ l BSA  
 15  $\mu$ l Buffer  
 64.9  $\mu$ l water  
 120  $\mu$ l total

Incubated in 37°C waterbath  
 2:50-4:50 heated inactivated  
 @ 65°C for 20 minutes

Conclusion: pkc15.1 cut with Spe I and Xho I.

Witnessed & Understood by me,

Date

Invented by

Date

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Objective: To amplify out the D0110 cell line's TCR chains by PCR. The  $\alpha$  chain will contain only the variable region with Sfi I and Sph I restriction sites for cloning. The  $\beta$  chain will contain the variable region and most of the constant region (ending just before the final cysteine) with Xba I and Sma I restriction sites for cloning.

Materials/Method: PCR buffer II  
 MgCl<sub>2</sub> sol'n  
 10mM dNTPs  
 pK16.7 (1:100)  
 pK18.7 (1:100)  
 purified water

KC114 (4 ports w/ Sfi I)  
 Jw TCR  $\alpha$  23 (2 back w/ Sph I) 10pm  
 KC115 (4 ports w/ Xba I)  
 KC118 (3 back w/ Sma I) 1/ul

Set up 10 reactions for each. Made 10  $\mu$ l pool and then added tag sites put in thermocycler once @ 96°C.

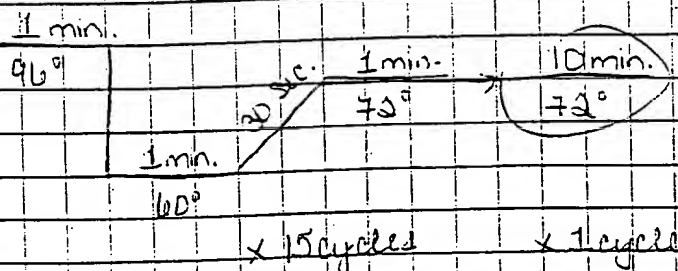
attached: 4.1 picture

$\alpha$	$\beta$
100 $\mu$ l buffer	"
80 $\mu$ l MgCl <sub>2</sub>	"
20 $\mu$ l dNTPs	"
10 $\mu$ l KC114	10 $\mu$ l KC115
10 $\mu$ l Jw TCR $\alpha$ 23	10 $\mu$ l KC118
20 $\mu$ l 16.7	30 $\mu$ l 18.7
755 $\mu$ l water	745 $\mu$ l water

lane 1 =  $\alpha$  Bst F II  
 2 =  $\alpha$  PCR (1/10 rxn)  
 3 =  $\beta$  PCR (1/10 rxn)

Conclusion: PCR worked!

+ tag (0.5  $\mu$ l / tube)



RE701-0-801-62 KC

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DNA

Objective: To pool and digest the PCR products to prepare for ligation.

Method/Materials: 2 PCR's C-801-62  
 A PCR's D-801-62  
 Sfi I @ 50u/l  
 Ssp I @ 15u/l  
 Xma I @ 10u/l  
 Xho I @ 50u/l  
 BSA (1:10)  
 Buffer 1  
 Buffer 2  
 purified water

Method: 2 digest  
 5ul Sfi I  
 7ul Ssp I  
 24ul BSA  
 24ul buffer 1  
 135ul water  
 45ul DNA  
 240ul  
 ↓

2 hours @ 37°C  
 2 hours @ 50°C

3 digest  
 10ul Xma I  
 5ul Xho I  
 30ul BSA  
 30ul buffer 1  
 180ul water  
 45ul DNA  
 300ul  
 ↓

4 hours @ 37°C

Heat inactivated @ 65°C for 20 minutes

Conclusion: Digests performed as described.

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Objective: To anneal a pair of oligos together to make the linker sequence for the single chain construct.

Materials/Method: JA301 @ 10 pm/ul  
JA302 @ 10 pm/ul  
2 annealing buffers

Mixed: 25 ul buffer added to thermocycler once @ 96°C.  
12.5 ul JA301  
12.5 ul JA302 3 min. 10 min.  
50 ul total 96°C

30 min.

Attached: Gel picture  
(30% success / 1% sequencing)

2 min.  
30°C

Want to see two oligos annealed.

RE701-0-801-70

lane 1 = MSP  
2 = JA301  
3 = JA302  
4 = annealed oligos

Conclusion: There appears to be annealed oligos present. The oligo insert is @ 25 pm/ul.

\* Want to convert oligo concentration from pm/ul to ng/ul.

$$165 \text{ bp} \times 1660 = 42900 \text{ g/mole} \\ \# 3 \times 10^4 \text{ pg/pm}$$

$$\frac{4.3 \times 10^4 \text{ pg}}{\text{pm}} \times \frac{25 \text{ pm}}{\text{ul}} = 1.08 \times 10^5 \text{ pg/ul} \\ \therefore 108 \text{ ng/ul}$$

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No. N/AObjective: to transform *X. laevis* cells with the ligated DNA from D-801-71

Materials: *X. laevis* competent cells  
 ligated DNA D-801-71  
 B17-1 media  
 B12-1 + amp plates

Same method as on D-801-50.

Conclusion: Colonies counted.

1:10 10  $\mu$ l = 48  
 100  $\mu$ l = 423

1:10 10  $\mu$ l = 116  
 100  $\mu$ l = 329

1:30 10  $\mu$ l = 38  
 100  $\mu$ l = 422

1:30 10  $\mu$ l = 9  
 100  $\mu$ l = 74

The numbers tend (high colony #  $\rightarrow$  low colony # as insect concentration rises) look promising.  
 Selected 40<sup>th</sup> 12 colonies from 100  $\mu$ l 1:30 plate and grew 5ml overnight cultures with amp + tetr. for each.

KC

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Objective: To pre and digest the V<sub>2</sub> PCR products to prepare for ligation.

Method/Materials: V<sub>2</sub> PCR products C-811e-15  
Sfi I @ 20ul  
Spe I @ 15ul  
Buffer 2  
BSA (1:10)  
purified water  
Qia spin PCR Purification kit

① PCR products pre and cleaned up using kit. Volume = 50ul

② X digest

7ul Sfi I  
7ul Spe I  
24ul BSA  
24ul Buffer 2  
135ul water  
45ul DNA  
240ul

Cut for 2 hours @ 37°C.  
Cut for 2 hours @ 50°C.

③ Heat inactivated for 20 minutes @ 65°C.

Conclusion: Digestion performed as described.

RC

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C-114

Objective: To gel purify and then quantitate the cut  $\lambda$  PCR products.Method/Materials:  $\lambda$  (114/119) x SpeI x SfiI 10-816-7

10x prep agarose gel

Qimquick Spin Gel Purification kit QIAGEN

① DNA was precipitated and resuspended in 30  $\mu$ l H<sub>2</sub>O TE buffer.  
 Ran prep gel with  $\lambda$  SfiI digest. Cut out fragment of interest.

② DNA purified according to manufacturer's instructions.

③ Ran 10 $\mu$ l gel to quantitate DNA.

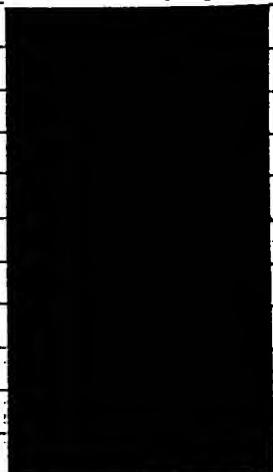
Gel: 1 =  $\lambda$  SfiI (2  $\mu$ l)  
 2 = " (2  $\mu$ l)  
 3 = " (2  $\mu$ l)  
 4 = " (2  $\mu$ l)  
 5 = DNA (5  $\mu$ l)

Conclusion: The 5  $\mu$ l of sample DNA  
 looks like the 2  $\mu$ l lane of the  
 minicolumn = 28 ng.  
 The DNA is @ 5.5 ng/ $\mu$ l.

Attached: gel pictures.

Lanes A+B are PCR products  
 100/116 and 100/118. See  
 10-816-19-20 for PCR reactions.

RE701-D-816-18



RE701-D-816-18



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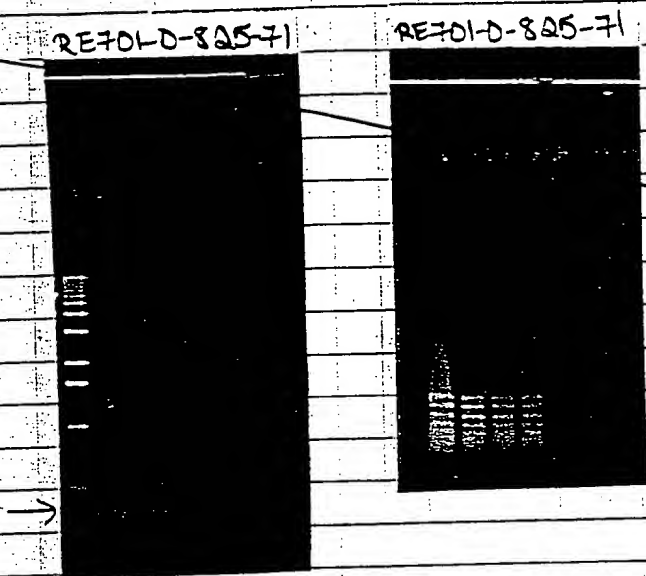
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Objective: to gel purify and quantitate 114/126 cut with Sfi I and Spe I

Method/materials: 114/126 x Sfi I x Spe I 0-825-71  
Qiaquick Spin Purification kit - Qiagen

✓ Ran DNA in 2% prep agarose gel. Isolated fragment of interest  
Purified DNA using kit.

Attached: Gel pictures.



For gel B, lane 1 = msp I (4ug)  
2 = " (2ug)  
3 = " (1ug)  
4 = " (0.5ug)  
5 = 114/126 (2ul)  
6 = " (3ul)

Conclusion: 5ul looks like 1/3 of the 3rd  
band down in the 0.5ug mwm lane

$\therefore \frac{404}{4361} = 92.6 \text{ ng/ug mwm}$   
 $\frac{4361}{2 \div 3} = 15.4 \text{ ng/5ul sample}$   
 $114/126 @ = 3 \text{ ng/ul}$

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Page No. N/A

Objective: To ligate 114/126 (V<sub>2</sub>) into pUC278

Materials: 114/126 x Sfi I x Spe I D-825-70 @ 300 µl  
 pUC278 x Sfi I x Spe I D-811-26 @ 300 µl  
 T4 ligase  
 T4 ligase buffers

Method: Set up 4 ligations

Vector ≈ 3500 bp. Insert ≈ 350 bp. Ratio = 1:10 (vector:insert)  
 Same method as on D-825-25 except incubated 1 hour @ room temperature.

	1:1	1:1	1:3	3:1
vector	44	44	44	131
insert	—	41	140	41
buffer	1	2	22	22
ligase	1	1	1	1
water	3.6	2.9	0.4	1
	10 µl	20 µl	22 µl	22 µl

Conclusion: Ligation set up as described.

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Objective: To transform  $\lambda$ 1B cells with pCR4.2 ligated DNA

Materials: pCR4.2 ligation 0-825-23  
 $\lambda$ 1B cells  
BLT-1 media

Method: Same as on 0-825-28, #① and ②

Conclusion: Colonies counted

1:10 10  $\mu$ l = 6  
100  $\mu$ l = 58

1:3 10  $\mu$ l = 388\*  
100  $\mu$ l = TMT

1:1 10  $\mu$ l = 243  
100  $\mu$ l = TMT

3:1 10  $\mu$ l = 187  
100  $\mu$ l = TMT

Conclusion: Transformation numbers close very good.  
Grew 10 colonies on \* plate

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Page No. 1A

Objective: To isolate plasmid DNA and perform analytical digestion on pKC42 candidates.

Materials/Method: Used Qiagen's Qiaprep Plasmid Extraction kit to isolate plasmid DNA from cell pellets of candidates.  
Made 12x pool of reagents:

3  $\mu$ l Sfi I (@ 20U/ $\mu$ l)  
3.6  $\mu$ l Spe I (@ 15U/ $\mu$ l)  
1.8  $\mu$ l Buffer 1  
1.8  $\mu$ l BSA (1:10)  
17.4  $\mu$ l water

→ 5  $\mu$ l to each + 10  $\mu$ l sample DNA (or 2  $\mu$ l vector DNA)

Incubated @ 37°C for 1 hour. Incubated @ 50°C for 1 hour.

Attached: Gel picture.

RE701-0-825-78 42.1-10

Lane 1 = Msp I (1  $\mu$ g)

2-11 = candidates # 1-10

12 = vector only (27.8)

Conclusion: 10/10 candidates have an insert of the correct size.

Selected #1, 2, and 3 to focus on. Streaked out for single colonies. Will prepare DNA for sequencing.

KE

To Page No. 1A

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Objective: To pool and digest the V<sub>6</sub>C<sub>3</sub> PCR products for ligation.

Method / Materials: V<sub>6</sub> PCR products (122/116) 2-816-20  
" (122/118) "

Xma I @ 100 U/ul

Xho I @ 20 U/ul

Buffer 1

BSA (1:10)

purified water

QiaSpin PCR Purification kit Qiagen

① PCR products pooled and cleaned using kit. Volume = 50 ul

② Digest (set up for 122/116 and 122/118 separately)

10 ul Xma I

5 ul Xho I

30 ul BSA

30 ul Buffer 1

180 ul water

45 ul DNA

300 ul

Cut yes 4 hours @ 37°C.

③ Heat inactivated 20 minutes @ 65°C.

Conclusion: Digestions performed as described.

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Objective: to gel purify and then quantitate the cut  $V_3$  PCR products.

Materials:  $V_3$  (122/116) x  $XhoI$  x  $XmaI$  0-816-24  
 $V_3$  (122/118) x  $XhoI$  x  $XmaI$  0-816-24  
Same as on 0-816-18.

Method: Same as on 0-816-18.

Hel: 1 = 7 BS+ET (2  $\mu$ g)  
2 = " (1  $\mu$ g)  
3 = " (0.5  $\mu$ g)  
4 = " (0.25  $\mu$ g)  
5 = 122/116 (5  $\mu$ l)  
6 = 122/118 (5  $\mu$ l)

Conclusion:

122/116 looks like the 2  $\mu$ g band  
 $\times 3 = 28ng \times 3 = 84ng$   
 $\therefore 1 \mu l = 11.8 ng$

122/118 looks like the 2  $\mu$ g band  
 $= 28ng$   
 $\therefore 1 \mu l = 5.6 ng$

Attached: Gel pictures

RE701-0-816-29

RE701-0-816-29

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Page No. N/A

Objective: To ligate  $V_3(122/116)$  with pRC24.2.  $V_3(122/116) = \beta$  chain w/ 1.2  $\mu$ g

Materials/Method:  $V_3(122/116) \times \text{XbaI} \times \text{KmaI}$  0-816-29 @ 16.8 ng/ $\mu$ l  
 pRC24.2  $\times \text{XbaI} \times \text{KmaI}$  @ 4.2 ng/ $\mu$ l  
 T4 ligase  
 T4 ligase buffer  
 purified water

① Same as step ① on 0-816-27

② Set up 4 ligations. Vector  $\approx 3900$ . Insert  $\approx 750$  bp. Ratio = 5:5

	1:1	1:1	1:3	3:1
vector	3.4 $\mu$ l (140 ng)	3.4	3.4	10.2
insert	1	15 $\mu$ l (255 ng)	4.5	1.5
buffer	1 $\mu$ l	1	1	2
ligase	1 $\mu$ l	1	1	1
water	4.6 $\mu$ l	3.1	2.1	5.3
	10 $\mu$ l	10 $\mu$ l	10 $\mu$ l	20 $\mu$ l

Conclusion: Ligation set up as described.

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To Page No. N/A

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Page No. N/A

Objective: To ligate  $V_p(122/118)$  with pKCS4.2 (pKCS30)  $V_p(122/118) \Rightarrow \beta$  chain 1001/1001

Materials/Method:  $V_p(122/118) \times \text{vhoT} \times \text{vhoAT}$  0-816-29 @ 5.6 ng/ul  
 pKCS4.2  $\times \text{vhoT} \times \text{vhoAT}$  @ 41.2 ng/ul  
 T4 ligase  
 T4 ligase buffers  
 purified water

① Same as step ① on 0-816-27

② Set up 3 ligations (1:6 was already set up on 0-816-30)  
 Vector  $\approx 3000$  bp. Insert  $\approx 700$  bp. Ratio = 5.5

	1:1	1:3	3:1
vector	3.4 $\mu$ l (140 ng)	3.4	10.2
insert	4.1 $\mu$ l	13.8	41.6
buffer	1 $\mu$ l	2	2
ligase	1 $\mu$ l	1	1
water	10 $\mu$ l	20.2 $\mu$ l	20.2 $\mu$ l

Conclusion: Ligations set up as described

To Page No. N/A

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Page No. N/A

Objective: To transform XLB cells with the ligated DNA from D-816-30 and D-816-31.

Materials: ligated DNA from D-816-30 (pUC29)  
 ligated DNA from D-816-31 (pUC30)  
 XLB cells  
 B17+ media  
 B12-1+amp. plates

Method: same as on D-816-28.

Conclusion: Colonies counted next day.

Picked 10 colonies from each plate to grow O.N.

1:1 10 $\mu$ l = 0  
 100 $\mu$ l = 9

pUC29

pUC30

1:1 10 $\mu$ l = 6  
 100 $\mu$ l = 97

1:1 10 $\mu$ l = 2  
 100 $\mu$ l = 316

1:3 10 $\mu$ l = 2  
 100 $\mu$ l = 162

1:3 10 $\mu$ l = 2  
 100 $\mu$ l = 42

3:1 10 $\mu$ l = 4  
 100 $\mu$ l = 79

3:1 10 $\mu$ l = 16  
 100 $\mu$ l = 41

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Page No. 1/4

Objective: To screen pKC29 and pKC30 candidates for inserts.

Method/notes: ① Used Qiaspin Plasmid Extraction Kit from Qiagen to isolate plasmid DNA.

② Cut 1/100 DNA with XbaI and XbaI to look for an insert of  $\approx 300$  bp. Made 24 pools:

- 6  $\mu$ l XbaI
- 10  $\mu$ l XbaI
- 10  $\mu$ l BSA (100)
- 10  $\mu$ l 3.12
- 7.5  $\mu$ l purified water
- 10  $\mu$ l

- ③ Incubated @  $37^{\circ}\text{C}$  for 2 hours
- ④ Ran 1% agarose gels. Lane 12 = control

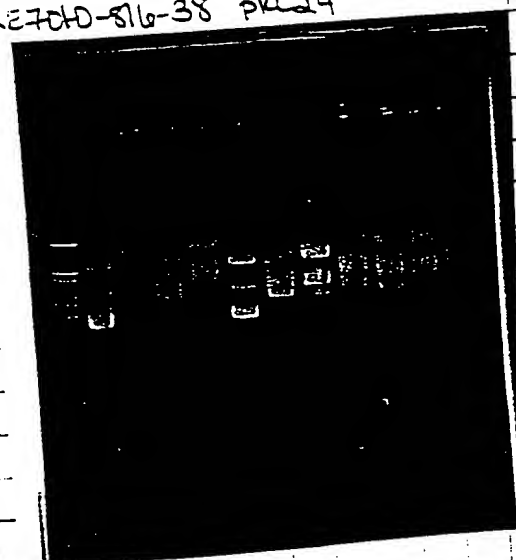
5  $\mu$ l to each digest  
 + 10  $\mu$ l DNA  
 or 5  $\mu$ l control  
 (control = pKC29)

Conclusion: For 29 (gel on left), 6/10 candidates have an insert of the correct size. Selected candidates # 2, 4, 8 and streaked out single colonies.

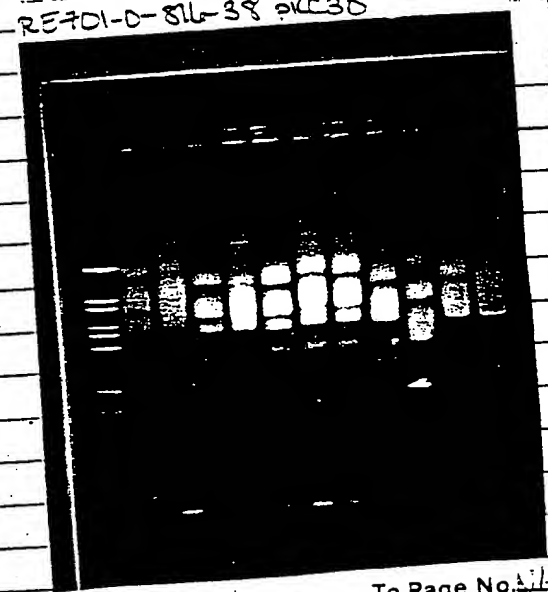
For 30 (gel on right), 6/10 candidates have an insert but candidate # 11's insert is longer than the other. Selected candidates # 2, 3, 6 and streaked out single colonies.

Attached: Gel pictures

RE701-0-816-38 pKC29



Attached: Gel picture  
 RE701-0-816-38 pKC30



To Page No. 1/4

Witnessed & Understood by me,

Date

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Objective: To digest pKC34.3 and pKC35.2 with XhoI and EcoRI.

Method/Materials: Decided to cut 20  $\mu$ g of DNA each.  
Set up digests

100  $\mu$ l pKC34.3 ( $\approx$  18  $\mu$ g total) D-825-67

5  $\mu$ l XhoI (@ 20 U/ $\mu$ l)

1  $\mu$ l EcoRI (@ 100 U/ $\mu$ l)

14  $\mu$ l Buffer 2

14  $\mu$ l BSA (1:10)

6  $\mu$ l water

140  $\mu$ l total

45  $\mu$ l pKC35.2 (20  $\mu$ g) D-825-67

5  $\mu$ l XhoI

1  $\mu$ l EcoRI

14  $\mu$ l Buffer 2

14  $\mu$ l BSA (1:10)

6  $\mu$ l water

140  $\mu$ l total

Incubated @ 37°C from 2-4 pm.

Heat inactivated @ 65°C for 20 minutes.

Precipitated DNA and resuspended in 50  $\mu$ l 1/10 TE buffer.

Conclusion: DNA digested as described.

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KC To Page No. N/A



Page No. N/A

Objective: To isolate plasmid DNA from pRC42.1, 2, and 3

Method/Materials: Same as on 0-825-45

Conclusion: OD scans were not printed out

pRC42.1 (1:100)      260 = 0.2053      260/280 = 1.59  
280 = 0.1288

$$0.2053 \times 50 \times 100 = 1026.5 \text{ ng/}\mu\text{l} \times 200 \mu\text{l} = 205.3 \mu\text{g}$$

pRC42.2 (1:100)      260 = 0.1896      260/280 = 1.78  
280 = 0.1063

$$0.1896 \times 50 \times 100 = 948 \text{ ng/}\mu\text{l} \times 200 \mu\text{l} = 189.6 \mu\text{g}$$

pRC42.3 (1:100)      260 = 0.1658      260/280 = 1.81  
280 = 0.0915

$$0.1658 \times 50 \times 100 = 829 \text{ ng/}\mu\text{l} \times 200 \mu\text{l} = 165.8 \mu\text{g}$$

Conclusion: DNA isolated as described

KC To Page No. N/A

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

To digest the pKC42 candidates #1, 2, 3 with XhoI and EcoRI

Materials: Decided to cut 20ug of each.  
Set up digests

100ul pKC42.1  
5ul XhoI (@ 50U/ul)  
1ul EcoRI (@ 100U/ul)  
12ul Buffer 2  
12ul BSA (1:10)  
70.5ul water

120ul total

211ul pKC42.2  
5ul XhoI  
1ul EcoRI  
12ul Buffer 2  
12ul BSA (1:10)  
108.4ul water

120ul total

214 1 ul pKC42.3  
5ul XhoI  
1ul EcoRI  
12ul Buffer 2  
12ul BSA (1:10)  
105.9ul water

120ul total

Incubated @ 37°C 4hr 10:40 am to 1:40 pm

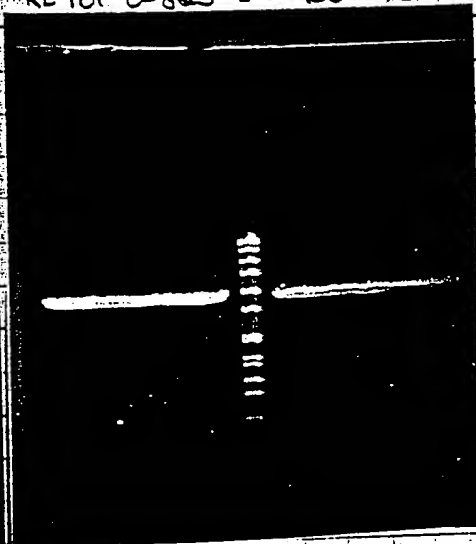
Heat inactivated @ 65°C for 20 minutes

Precipitated DNA and resuspended in 50ul 1/10 TE buffer

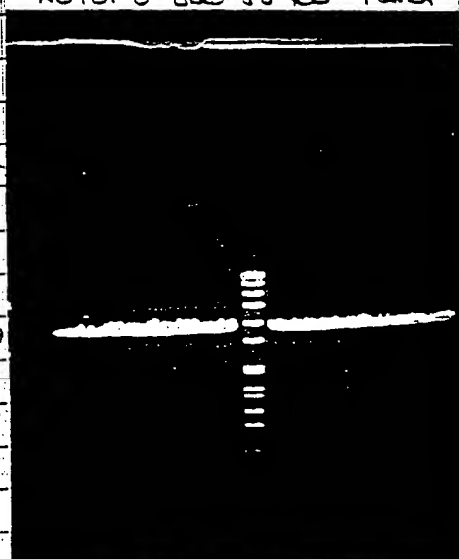
Ran DNA in 1% prep agarose gels. Cut out fragments of interest and probe

Attached: Gel pictures. Conclusion: DNA digested as described.

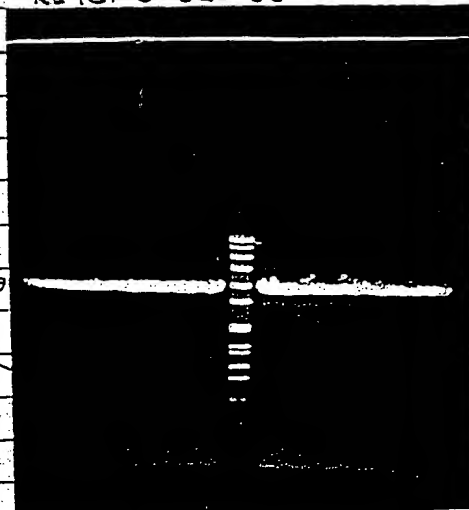
RE701-D-825-88 KC 42.1



RE701-D-825-88 KC 42.2



RE701-D-825-88 KC 42.3



To Page No. 17A

Witnessed & Understood by me,

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Page No. N/A

Objective: To gel purify and then dephosphorylate pKC42 1, 2, 3 digested with XbaI and EcoRI.

Method/Materials: pKC42 1, XbaI, EcoRI 0-825-88  
pKC42.2 " "  
pKC42.3 " "  
Qiaquick spin gel <sup>Extraction</sup> Purification kit - Qiagen

① Purified DNA out of isolated gel fragments on 0-825-88 using kit. Final volume = 100  $\mu$ l each.

② Dephosphorylated each:

100  $\mu$ l DNA  
1  $\mu$ l CIAP  
12  $\mu$ l Buffer 12  
7  $\mu$ l water  
120  $\mu$ l total

Incubated @ 37°C from 12:25pm to 2:05pm.

Cleaned up using Qiaquick spin PCR Purification kit.  
Final volume = 100  $\mu$ l each.

Will quantitate cut DNA after the correct clone has been identified by sequencing [see 0-825-914] K.C. and

Conclusion: DNA was gel purified and dephosphorylated.

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To Page No. N/A

TITLE Ligation of  $\beta$ -gene VIII into pKC42.3  
(pKC43 + 44)

4

From Page No. N/A

\* For previous work on this project, please see DE701-D-825

Objective: To ligate  $\beta$ -gene VIII into pKC42.3 to yield 2 single chain constructs

Materials: pKC42.3 vector EORI @ 4.2  $\mu$ l (0-825-93)  
 pKC34.3 " " @ 8.8  $\mu$ l (0-825-74)  
 pKC35.2 " " @ 11.8  $\mu$ l (0-825-74)  
 T4 ligase  
 T4 ligase buffer

Method: Set up <sup>3</sup>pc ligations with pKC34 and <sup>3</sup>pc with pKC35  
 plus 1 vector only ligation.

Vector  $\approx$  3400 bp. Insert  $\approx$  850bp Ratio = 1:4 (vector:insert)

Mixed all components except ligase. Heated 2 minutes @ 65°C.

Returned to ice, added ligase, and incubated 1 hour @ room temp.

43 (w/34.3)	1:0	1:1	1:3	3:1
vector	3.4	3.4	3.4	10.2
insert	—	4	12	4
buffer	1	1	2	2
ligase	1	1	1	1
water	4.6	0.6	1.6	2.8
	10 $\mu$ l	10 $\mu$ l	20 $\mu$ l	20 $\mu$ l

44 (w/35.2)	1:1	1:3	3:1
vector	3.4	3.4	10.2
insert	3	9	3
buffer	1	2	2
ligase	1	1	1
water	1.6	4.6	3.8
	10 $\mu$ l	20 $\mu$ l	20 $\mu$ l

Conclusion: Ligation set up as described.

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Invented by

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To Page No. N/A



Form Page No. N/AObjective: To transform XL-1B cells with pUC43 and pUC44

Materials: pUC43 ligation D-837-1  
 pUC44 " "  
 XL-1B cells  
 B17-1 media

Method: ① Chilled tubes on ice. Added 100  $\mu$ l cells and 1/2 of each ligation.  
 Let sit on ice for 30 minutes.

② Heat shocked @ 42°C for 45 seconds. Returned to ice.  
 Added 1 ml media to each. Let grow @ 37°C with shaking for 1 hour.  
 Plated 10  $\mu$ l and 100  $\mu$ l of each on B17-1 + Amp plates. Grown over-  
 night @ 37°C.

Conclusion: Colonies counted

pUC43 1:1 10  $\mu$ l = 0  
 100  $\mu$ l = 0

1:1 10  $\mu$ l = 74  
 100  $\mu$ l = TMC

1:3 10  $\mu$ l = 105\*  
 100  $\mu$ l = 105 TMC

3:1 10  $\mu$ l = 105  
 100  $\mu$ l = TMC

pUC44 1:1 10  $\mu$ l = 103  
 100  $\mu$ l = TMC

1:3 10  $\mu$ l = 136\*  
 100  $\mu$ l = TMC

3:1 10  $\mu$ l = 139  
 100  $\mu$ l = TMC

Conclusion: Numbers look good!  
 Used 10 candidates each on plates  
 marked with \*

To Page No. N/A

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

Objective: To induce the Fab constructs containing the DO11.10 cell line's TCR (pKC34, 35, 40, and 41) and the SC constructs (pKC43 and 44). pKC18 is a positive control for the  $\beta$  chain.

Materials:

cells all adapted to high phosphate media  
 high phosphate media      low phosphate media  
 protein extraction buffer (R77)      urea

Method: ① Cells grown overnight in high phosphate media @ 30°C with shaking - 5ml each.

Attached: Basic induction protocol.

INDUCTION OF THE *phoA* PROMOTER SYSTEM:

Small scale:

Grow overnight culture in high phos. medium (B5) @ 30°C... 5 mls is enough

② Harvest <sup>1</sup>/<sub>2</sub> mls into each of two tubes...

Pellet cells (gently)... wash one pellet with high phos. medium, and the other with low phos. medium (B8)...

Repeat once...

Resuspend the appropriate pellet with <sup>1</sup>/<sub>2</sub> mls high phos. medium, and the other with <sup>1</sup>/<sub>2</sub> mls low phos. medium... → only for some constructs:

Inoculate the resuspended cells into <sup>50</sup>/<sub>100</sub> mls of the appropriate medium... see below

Induction is carried out with growth @ 30°C for 4+ hours - overnight.

- NOTE: Both B5 and B8 already contain ampicillin !

Inductions were carried out for 5 hours or overnight as noted:

5 hr. → 34, 35, 40, 41, 43, 44, 18

5 hr. non-induced → 44

ON → 34, 35, 40, 41, 43, 44, 18

ON non-induced → 44, 18

## Induction of Fab and single chain constructs 2

③ OD<sub>600</sub>s were measured:

34-5 ( $0.04 \times 2 = 0.08$ )	→ use all 50ml	= 10 ODs
35-5 ( $0.07 \times 2 = 0.14$ )	"	"
40-5 ( $0.15 \times 5 = 0.75$ )	13.3 ml	"
41-5 ( $0.1 \times 5 = 0.5$ )	20.0 ml	"
43-5 ( $0.03 \times \text{neat} = 0.03$ )	all	"
44-5 ( $0.1 \times 5 = 0.5$ )	20.0 ml	"
18-5 ( $0.12 \times 5 = 0.6$ )	16.7 ml	"
44N-5 ( $0.055 \times 10 = 0.55$ )	18.2 ml	"
34-ON ( $0.09 \times 10 = 0.9$ )	11.1 ml	"
35-ON ( $0.21 \times 10 = 2.1$ )	4.8 ml	"
40-ON ( $0.09 \times 10 = 0.9$ )	11.1 ml	"
41-ON ( $0.11 \times 10 = 1.1$ )	9.1 ml	"
43-ON ( $0.05 \times 10 = 0.5$ )	20.0 ml	"
44-ON ( $0.26 \times 10 = 2.6$ )	3.8 ml	"
18-ON ( $0.1 \times 10 = 1.0$ )	10.0 ml	"
44N-ON ( $0.4 \times 10 = 4.0$ )	2.5 ml	"
18N-ON ( $0.37 \times 10 = 3.7$ )	2.7 ml	"

④ Cells (volumes listed for 10 ODs) were spun down. Pellets resuspended in 1ml protein extraction buffer. Sonicated 2 minutes using cup sonicator. Spun 5 minutes @ maximum speed. Supe. = soluble fraction. Resuspended pellets in 1ml of same buffer + 8M urea. Rocked 1/2 hour @ room temperature and spun 5 minutes @ maximum speed. Supe. = insoluble fraction.

Western Blot + Coomassie blue of dygates

Objective: to look @ E coli dygates for the induction of either Fab TCR or single chain TCR.

Materials: dygates prepared on p. 4-5  
cracking buffer w/ same sheets  
12.5% SDS-PAGE gels membrane

Method: ① dygates were diluted 1:2 in cracking buffer (10ml dygate + 10ml buffer, per lane)  
Boiled 5 minutes. Run out on 12.5% gels.

② Transferred to membrane for 15 minutes @ 250-300 mamps.

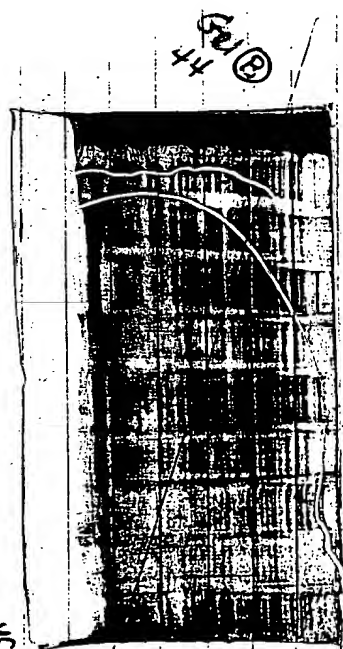
③ Blebbed 1 hour in blotto. diluted 1:10 in 1x PBS. Washed 1x. Probed for 1 hour with  $\alpha$ - $\gamma$ 3 TCR-HRP  
④ 1:1.5K (36 $\mu$ l / 39ml buffer) in blotto diluted 1:50 in 1x PBS. Washed 3x. Exposed to ECL-HRP substrate for 1 minute. Exposed film.

Attached: Coomassie stained-gel.



Western Blot + Coomassie blue of dygates

attached: Coomassie blue-stained gel.

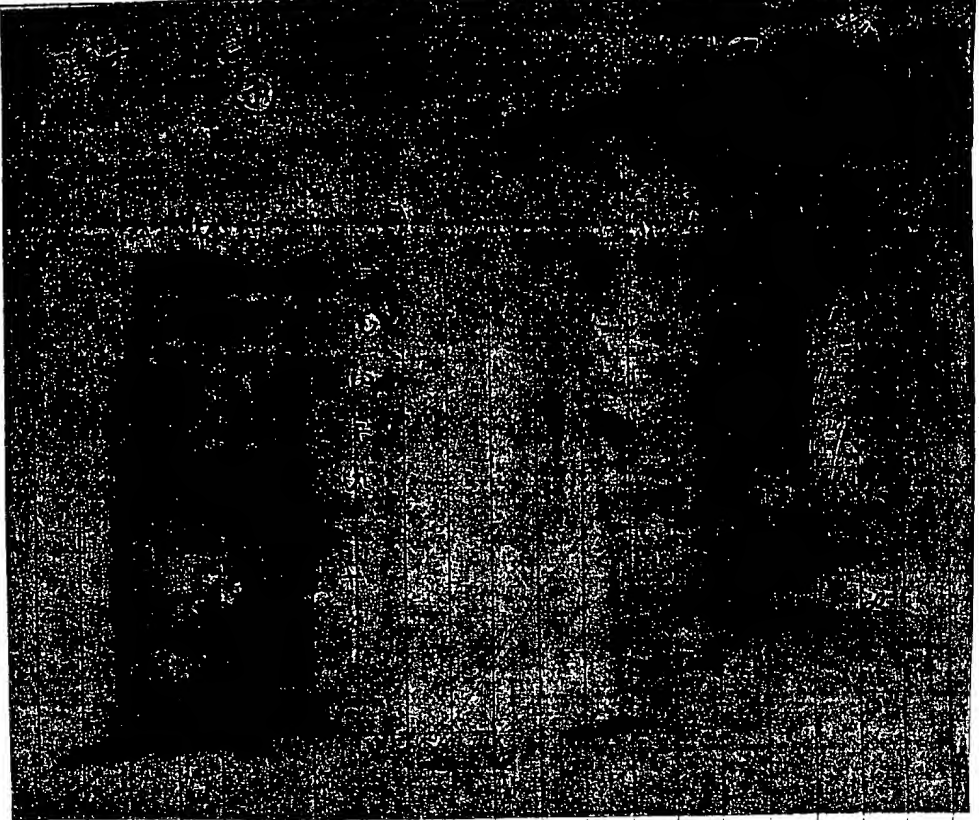


attached: Blot. 10 minute exposure.



Western Blot + Coomassie Gel of dygates

Attached: Blot. 1 hour exposure.



Blot A

- |                    |                     |
|--------------------|---------------------|
| 1) mwm             |                     |
| 2) 43 I - 5 h. (S) | 10) 18 I - 5 h. (S) |
| 3) " (I)           | 7) " (I)            |
| 4) 43 I - DN (S)   | 8) 18 I - DN (S)    |
| 5) " (I)           | 9) 18 NI - DN (S)   |
|                    | 10) 18 NI - DN (I)  |

Western Blot + Coomassie Gel of dygates

Blot B

- |                    |                     |
|--------------------|---------------------|
| 1) mwm             | 6) 44 NI - 5 h. (S) |
| 2) 44 I - 5 h. (S) | 7) " (I)            |
| 3) " (I)           | 8) 44 NI - DN (S)   |
| 4) 44 I - DN (S)   | 9) " (I)            |
| 5) " (I)           | 10) 18 I - DN (I)   |

I = induced  
NI = non-induced  
DN = overnight

(S) = soluble  
(I) = insoluble

Conclusion: pUC44 has a lot of material in the overnight, insoluble fraction. It is the right size (~50 kD) and may be in the inner membrane, which is where we would like to see it. The other constructs do not show induction.

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Purification of SCTER using  $\alpha$ - $\frac{1}{3}$  8.2  
Antibody column (SCTER lot #5)

Objective: Want to try purifying the SCTER  
using an  $\alpha$ - $\frac{1}{3}$  8.2 affinity column.

Method: ① started with 22.30g of yeast extract paste  
from 1 suspension. lot = 0-758.91, yeast extract #2,  
9/13/05.

Decided to resuspend paste @  $\approx 100$  ml in buffer.  
(Buffer = 50mM Tris, 150mM NaCl, 0.5% NP40)

$\approx 3$  liters of culture = 130 grams of cell paste

OD =  $30.7 \pm 1$  ml

so decided to resuspend paste in 150ml

$$\frac{3000 \text{ ml}}{130 \text{ g}} = \frac{x}{22.30 \text{ g}} \quad x = 516 \text{ ml} \times 30.7 \text{ OD/ml} =$$
$$15,841.2 \text{ OD} = 105 \text{ L OD/ml}$$
$$150 \text{ ml}$$

To the 150 ml of buffer, added 3 tablets of "Complete"  
(proteinase inhibitor from Boehringer Mannheim). Set  
dissolve completely.

② Resuspended cell paste in buffer. Passed through  
French press twice.

③ Set solubilize @  $4^\circ\text{C}$  with shaking / rocking for  
 $\approx 4 \frac{1}{2}$  hours.

④ Spin to soluble fraction, added  $\text{NaBr}_2$  -  $\alpha \frac{1}{3}$  8.2  
resin (see pages 7-3) and set rock overnight  
@  $4^\circ\text{C}$ .

Purification of SCTER

⑤ Split sample into 3-50ml tubes. Spin gently  
@ 800 rpm for 2 minutes to pull down resin.  
loaded tube as "yellow tube"

⑥ Pooled resin together in 1 tube and washed 3x  
with more buffer. Transferred resin to column  
and washed 1x with more buffer.

⑦ Eluted two fractions:  
fraction #1 = 1ml of 0.1M glycine + 0.1% NP40  
fraction #2 = 2ml " , pH 3.0

neutralized each fraction with 200ul ammonia, pH 8.0.

⑧ Split fraction #2 among four microcon-100 concentrators.  
Spin 20 minutes @ maximum speed  
@  $4^\circ\text{C}$ . Wash = add 300ul super buffer (R50) +  
0.1% NP40 and spin as above. Washed 3x.

⑨ Pooled sample from four tubes to two tubes  
and rinsed membranes. Washed 3x.

⑩ Pooled sample from two tubes to 1 tube. Rinsed  
membranes and washed 3x. Rinsed membrane  
checked pH etc. Final volume = 240ul in heped  
buffer + 0.1% NP40.

Conclusion: SCTER prep. (lot #5) prepared.

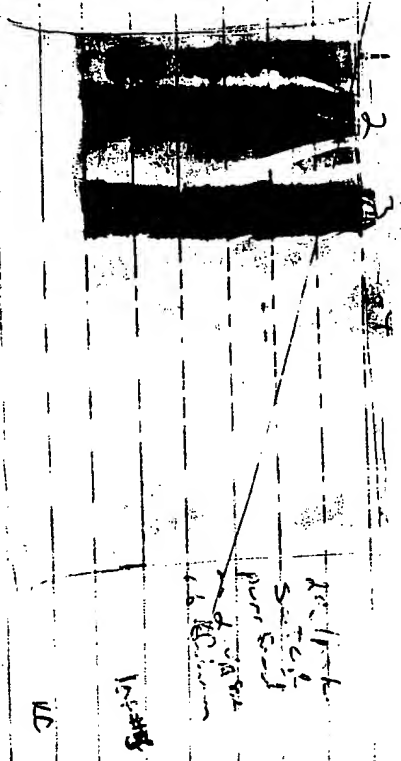
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Comassie Gel of Purified SETR Lot #5

Objective: To look @ the purified SETR.  
This lot was purified with the  $\alpha$ - $\gamma$ 8.2 Ab.

Method: Same as on page 74 but did not run a Western blot.

Attached: Comassie stained gel.



1 = MW marker  
2 = IgG  
3 = IgG + 18 uL / 240 uL total  
4 = purified SETR  
5 = purified SETR

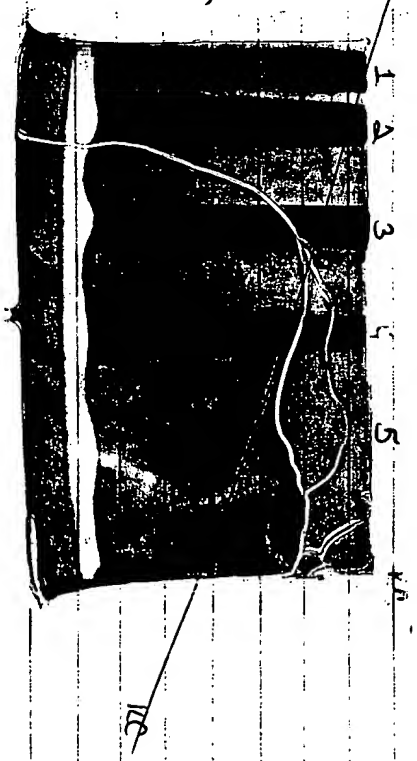
Conclusion: Can see purified SETR band!

Western Blot + Comassie Gel of SETR (Lot #6)

Objective: To look @ the purified SETR.  
This lot was purified with both the  $\alpha$ - $\gamma$ 8.2 and  $\alpha$ - $\gamma$ 5TER Ab columns combined.

Method: Same as on page 74.

Attached: Comassie stained gel.



1 = MW marker  
2 = IgG  
3 = IgG + 18 uL / 240 uL total  
4 = IgG + 18 uL / 240 uL total  
5 = purified SETR

Conclusion: Purified SETR looks good!